

CD44 Isoforms Containing Exon V3 Are Responsible for the Presentation of Heparin-binding Growth Factor

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Abstract. Glycosaminoglycan-modified isoforms of CD44 have been implicated in growth factor presentation at sites of inflammation. In the present study we show that COS cell transfectants expressing CD44 isoforms containing the alternatively spliced exon V3 are modified with heparan sulfate (HS). Binding studies with three HS-binding growth factors, basic-fibroblast growth factor (b-FGF), heparin binding-epidermal growth factor (HB-EGF), and amphiregulin, showed that the HS-modified CD44 isoforms are able to bind to b-FGF and HB-EGF, but not AR. b-FGF and HB-EGF binding to HS-modified CD44 was eliminated by pretreating the protein with heparitinase or by blocking with free heparin. HS-modified CD44 immunoprecipitated from keratinocytes, which express a CD44 isoform containing V3, also bound to b-FGF. We examined whether HS-modified CD44 isoforms were expressed by activated endothelial cells where they might present HS-binding growth factors to leukocytes during an inflammatory response. PCR and antibody-binding studies showed that activated cultured

endothelial cells only express the CD44H isoform which does not contain any of the variably spliced exons including V3. Immunohistological studies with antibodies directed to CD44 extracellular domains encoded by the variably spliced exons showed that vascular endothelial cells in inflamed skin tissue sections do not express CD44 spliced variants. Keratinocytes, monocytes, and dendritic cells in the same specimens were found to express variably spliced CD44. $^{35}\text{SO}_4^{-2}$ -labeling experiments demonstrated that activated cultured endothelial cells do not express detectable levels of chondroitin sulfate or HS-modified CD44. Our results suggest that one of the functions of CD44 isoforms expressing V3 is to bind and present a subset of HS-binding proteins. Furthermore, it is probable that HS-modified CD44 is involved in the presentation of HS-binding proteins by keratinocytes in inflamed skin. However, our data suggests that CD44 is not likely to be the proteoglycan principally involved in presenting HS-binding growth factors to leukocytes on the vascular cell wall.

CD44 represents a very heterogeneous class of molecules all encoded by a single gene. Genomic cloning of CD44 has revealed that there are 19 exons (47), 12 of which can be alternatively spliced. At least 18 different CD44 transcripts have been identified and the potential for many more exists (for review see 35). The diversity of CD44 is further magnified by the differential use of numerous N-linked and O-linked glycosylation sites as well as glycosaminoglycan (GAG)¹ attachment sites. We

have become interested in whether these numerous isoforms provide for distinct and/or multiple CD44 functions.

A number of functions have been attributed to CD44, but functional characterization of CD44 isoforms has been limited. The extracellular domain of CD44 clearly functions as a cellular adhesion molecule, capable of binding extracellular matrix components including hyaluronic acid (1, 8, 41), fibronectin (24) and collagen (62), and mediating homotypic cellular aggregation (50). Its intracellular domain associates with cytoskeletal proteins ankyrin, actin, ezrin, radixin, and moesin (22, 31, 54, 59), and these interactions are believed to be critical for HA binding and CD44-dependent cellular mobility on plastic plates coated with HA (34, 56). CD44 expression on lymphocytes has been shown to be involved in hematopoiesis (41), selective homing to mucosal lymphatic tissue (25), and lymphocyte infiltration into cutaneous delayed type hypersensitivity sites in mice (5). Expression of CD44 may also be important during tumor growth and metastasis (10, 52). CD44 has been implicated in signal

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1. *Abbreviations used in this paper:* ACD, allergic contact dermatitis; AR, amphiregulin; b-FGF, basic-fibroblast growth factor; CS, chondroitin sulfate; GAG, glycosaminoglycan; HAEC, human aortic endothelial cells; HB-EGF, heparin binding-epidermal growth factor; HEP, heparitinase; HS, heparan sulfate; HUVEC, human umbilical vein endothelial cells; NHEK, normal human epithelial keratinocytes; OM, oncostatin; PSO, psoriasis; RT-PCR, reverse transcription polymerase chain reaction.

transduction. For example, cocross-linking of CD44 with either CD2 or the T cell receptor results in enhanced T cell proliferation, induced IL-2 receptor expression, IL-2 production, and Ca²⁺ flux (18, 46). Signaling induced by CD44 specific HA binding in macrophages has also been shown (42).

Proteoglycans have been shown to act as reservoirs for growth factors in many tissues (for review see 55, 63). Characterization of the biological significance of the interaction between heparan sulfate (HS) and GAG-binding growth factors has been demonstrated for the fibroblast growth factors (FGF) (45, 64), platelet-derived growth factor (PDGF) (43, 44), vascular endothelial growth factor (VEGF) (9), amphiregulin (AR) (7, 49), and heparin binding-epidermal growth factor (HB-EGF) (16, 17). Binding of growth factors to proteoglycans via GAG moieties has been proposed to provide a mechanism for growth factor recruitment at the cell surface, presentation to specific receptors, regulation of their action on target cells at short range, and establishment of a growth factor gradient within a tissue. These observations are consistent with the hypothesis that GAG-modified CD44 isoforms might be involved in growth factor, cytokine, and/or chemokine presentation. This view is supported by an *in vitro* model system showing that CD44 isolated from monocytes can present macrophage inflammatory protein-1 β (MIP-1 β) to T cells (53).

Proteoglycan-mediated presentation of chemokines to leukocytes has been proposed to play a major role during inflammatory responses. Leukocyte adhesion to endothelial cells at sites of inflammation has been modeled as a three step process described as follows: selectin mediated tethering and rolling, leukocyte activation, and activation-dependent tight cell adhesion which leads to diapedesis (for recent reviews see 4, 32, 33, 48, 65). It has been suggested that the leukocyte activation step involves proteoglycan-mediated chemokine presentation by activated endothelial cells lining the inflamed vascular wall. This mechanism of chemokine presentation permits the establishment of a chemokine gradient along and across the endothelium and prevents the chemokines from being swept away in the blood stream. GAG-modified CD44 isoforms expressed by vascular endothelial cells have been proposed to participate in the presentation of chemokines to leukocytes at sites of inflammation (53).

Some CD44 isoforms contain heparan sulfate, a glycosaminoglycan known to bind to a wide range of growth factors, cytokines, and chemokines. Here we define which CD44 isoforms are modified with HS, demonstrate the ability of HS-modified CD44 to bind to different HS-binding growth factors and identify cell types that use HS-modified CD44 for growth factor presentation.

Materials and Methods

Cell Culture

Normal human epithelial keratinocytes (NHEK), human umbilical vein endothelial cells (HUVEC), and human aortic endothelial cells (HAEC) were all purchased from Clonetics and grown in Clonetics media (San Diego, CA). All primary cells were used between passage 4–8. The fibrosarcoma cell line HT-1080 cells, WI-38 cells (fibroblasts), and COS cells were purchased from American Type Culture Collection (Rockville, MD) and grown in DMEM/10% FBS. Cells were treated with the following proinflammatory

mediators: 20 ng/ml TNF- α (R&D Systems, Minneapolis, MN), 200 U/ml IFN- γ (R & D Systems), 1 μ g/ml LPS (Sigma Immunochemicals, St. Louis, MO), 10⁻⁴ M histamine (Sigma Immunochemicals), 20 ng/ml IL-1 (R & D Systems), 2 U/ml thrombin (Sigma Immunochemicals), 20 ng/ml TGF- β (BMS-PRI, Seattle, WA), 20 ng/ml b-FGF (R & D Systems), and 20 ng/ml α -FGF (R & D Systems).

Construction of CD44-Ig Expression Vectors

A cassette vector was prepared in order to make soluble immunoglobulin fusion proteins of CD44 containing the variably spliced exons. Restriction sites for cloning in the alternatively spliced exons were placed into a CD44H full-length vector (pBCKS/FLCD44) as described by Jackson et al. (20). Using pBCKS/FLCD44 as template, the extracellular domain of CD44 was PCR amplified with primers designed to allow PCR products to be digested with HindIII and SphI. In a separate reaction, cDNA encoding a human IgG1 hinge and constant domains were PCR amplified from a 41BB-Ig expression vector (6), with primers designed to allow PCR products to be digested with SphI and XbaI. In a three way ligation, these two PCR fragments were ligated into HindIII-XbaI cut pBCKS/FLCD44 vector. The PCR oligonucleotides used for amplifying CD44H were the T3 primer and 44sphI-RP (GATCAGGCATGCTGG-AATTGGGATGTCCT-TATAGG). Amplification of the IgG domains was carried out with oligonucleotides R γ ISphI-FP (GCAGTCGCATGGAGCCCAAATCTTGTGAC) and CDM7-XBAI-RP (CCAATTATGTACACCCACAGAAGT-AAGG). The PCR conditions consisted of 6 min at 94°C followed by 25 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. Taq polymerase and reaction condition were used as suggested by the vendor (Perkin Elmer Cetus, Norwalk, CT). This CD44 expression cassette was used for cloning in the different CD44 variably spliced exons as described by Jackson et al. (20). To verify the absence of PCR-induced mutations all constructs were sequenced.

Metabolic Labeling and Enzymatic Digestion

Approximately 10⁷ cells were labeled with 1 mCi [³⁵S]NaHSO₄ (New England Nuclear, Boston, MA) for 4 h except when stated differently in the text. COS cell-generated CD44-Ig was produced by using a DEAE-dextran procedure and the fusion proteins were purified from tissue culture supernatants as described by Aruffo et al. (1). CD44 from cell lines was extracted in a lysis buffer containing 0.1 M NaCl, 0.01 M Na₂PO₄, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 μ g/ml of each of the protease inhibitors aprotinin, leupeptin, and pepstatin. Samples were precleared with 25 μ l protein G Sepharose for 1 h on ice. After the protein G Sepharose was spun out, 25 μ g/ml of Pgp-1 (PharMingen, San Diego, CA) was added and incubated on ice overnight. Protein G Sepharose was added for 2 h, and then washed six times in cold lysis buffer. Enzymatic digestions were carried out in the presence of the protein G Sepharose. The ³⁵SO₄-CD44 coupled to protein G was incubated for 1 h at 37°C with either 1 mU of *Flavobacterium heparinum* Heparitinase, or 50 mU of *Proteus Vulgaris* Chondroitin ABC lyase (ICN Immunobiologicals, Lisle, IL), or both. All samples were heated for 10 min at 95°C in 2 \times loading buffer with β -mercaptoethanol and ran on either 4% SDS-PAGE gels, or on Tris/glycine 4–12% gradient gels (Novex, San Diego, CA).

Iodination

1.5 μ g of b-FGF (R & D Systems), AR (BMS-PRI), HB-EGF (R & D Systems), and Oncostatin M (OM) (BMS-PRI) each in 20 μ l of PBS, were added in 20 μ l of 2 M KPO₄ pH 7.4, 300 μ Ci of ¹²⁵I (3 μ l), and then 3 μ l of 0.25 mg/ml Chloramine T was added to each sample, and incubated for 2 min at RT. The reaction was quenched by adding sequentially 1 μ l 20 mg/ml sodium metabisulfite, 14 μ l 300 mg/ml potassium iodide, 14 μ l 20 mg/ml *N*-glycyl-tyrosine, and incubated at room temperature for 2 min. The mixture was then passed over a PD-10 column (Pharmacia LKB Biotechnology, Piscataway, NJ) and fractions were eluted with 0.5 ml of 2 mg/ml BSA in PBS.

mAbs

Anti-CD44 mAb A3D8 and A3D8-FITC (Sigma Immunochemicals) were added at manufacturer's recommended concentration for FACS analysis. The anti-CD44V3 specific mAb was purchased from R & D Systems and used at 10 μ g/ml. CD44 immunoprecipitations were carried out with the anti-CD44 mAb pgp-1 at 10 μ g/ml (PharMinger, San Diego, CA). The ELAM-1 mAb was purchased from Becton Dickinson (Mountain View,

CA) and used at 10 $\mu\text{g}/\text{ml}$. Isotype-matched controls included G19.4-FITC, an IgG1 mouse mAb provided by Jeff Ledbetter (BMS-PRI), and mouse IgG1, IgG2a, IgG2b antibodies to plant proteins were purchased from Sigma Immunochemicals. Anti-mouse-FITC (Tago, Burlingame, CA) was used at 1:100. The polyclonal Ab raised against the CD44 variably spliced exons (rabbit IgG) and mAb VFF7, VFF8 against CD44V5 and CD44V6 (mouse IgG) have been described previously (15, 29) and were kind gifts of K.-H. Heider and G. Adolf (Bender ImmuneSystems, Vienna, Austria).

FACS Analysis

Cells were lifted from plates with EDTA, washed, and added to staining media (RPMI/2% FBS/0.1% azide). The cells were stained with the indicated antibodies for 30 min at 4°C. The cells were washed two times with RPMI and FITC-labeled secondary antibodies were added for 30 min at 4°C and washed two more times. The cells were fixed in 2% formaldehyde/PBS and analyzed on a Hewlett Packard FACScan. Expression of CD44 protein by cultured endothelial cells was calculated by taking the mean fluorescence intensity of isotype-matched controls divided into the mean fluorescence of the experimental data point to give the fluorescence shift for stimulated and unstimulated cells (Fig. 4). The fluorescence shift (level of expression) of CD44 on unstimulated cells was set to 100%.

Reverse Transcriptase-PCR

cDNA for PCR was prepared by a random primer method. 10 μg of total mRNA was incubated with 1 μl of 0.1 M Hexamer (GIBCO BRL) for 10 min at 65°C. Then 4 μl of 5 \times first strand buffer (GIBCO BRL), 0.1 M DTT (GIBCO BRL), 2.5 M dNTP's (Boehringer Mannheim Corp.), and 1 μl superscript RT (GIBCO BRL) was added and incubated for 1 h at 37°C. Thirty μl of dH₂O was added, and then 3 μl of the reaction volume was used for each PCR reaction. The PCR reactions were carried out in a total volume of 50 μl with the following reagents added together: 3 μl of cDNA, 3 μl 1.25 mM dNTP's (Boehringer Mannheim Corp.), 2.5 μl of each oligonucleotide (10 μM), 5 μl 10 \times buffer, and 0.5 U *Taq* DNA polymerase (Boehringer Mannheim Corp.). The oligonucleotides used as PCR primers were: (a) CD44E3-FP, GGGGTGTACATCCTCACATC; (b) CD44E16-RP ACTCCAACCTTCTTGACTCC; (c) CD44E7-FP,CTCAGCAGGCTGG-GACGG. The PCR program was as follows: 94°C for 5 min; 35 cycles were carried out at 94°C for 30 s, 54°C at 1 min, 72°C at 1 min 45 s.

Ligand Blot

2 μg of each recombinant CD44-Ig fusion protein were separated by electrophoresis on a Tris/glycine 4–12% gradient gel (Novex, San Diego, CA), and transferred onto nitrocellulose. The membrane was blocked overnight at 4°C with 3% BSA 0.1% Tween-20 in PBS. The membrane was washed twice for 5 min in PBS, and then ~ 12 μCi of the iodinated proteins were added in the blocking buffer for 1 h at RT. The nitrocellulose was washed five times in PBS 0.1% Tween-20 at RT, and then exposed to a phosphorimager screen overnight. The binding between the iodinated growth factors and the HS-modified CD44 isoforms were blocked with heparin (Porcine Intestinal mucosal heparin; Sigma Immunochemicals) by preincubating 300 $\mu\text{g}/\text{ml}$ of heparin with the growth factor for 30 min before adding the mixture to the membrane. The heparin-binding control involved incubating 2 μCi of each iodinated protein with either Sepharose or heparin/Sepharose (Sigma Immunochemicals). The Sepharose beads were washed five times in PBS at RT and the radioactivity measured in a gamma counter.

ELISA

Three concentrations of CD44V3V8-V10-Ig untreated and CD44V3V8-V10-Ig digested with 4 mU of *Flavobacterium heparinum* Heparitinase were immobilized to a Immulon 2 plate (Dynatech Laboratories, Chantilly, VA) in PBS overnight. The plate was blocked for 2 h with 1 \times Specimen Diluent (Genetic Systems, Seattle, WA). After washing the plate with 0.05% Tween-20/PBS, 0.8 $\mu\text{g}/\text{ml}$ of biotin-conjugated b-FGF (Boehringer Mannheim Corp.) was added to the wells in the absence and presence of 3 mg/ml heparin (Porcine Intestinal mucosal heparin; Sigma Immunochemicals) and incubated for 1 h at RT. The plate was washed, and then avidin-conjugated HRP (Sigma Immunochemicals) diluted with blocking solution 1:500 was added to the wells for 30 min at RT. The plate was washed and incubated with 100 μl of EIA Chromogen Reagen (Genetic Systems) for 10 min at RT. The reaction was stopped by addition of 100 μl of 1 N H₂SO₄ and the optical density measured on an ELISA reader at dual wavelengths 450 and 630 nm.

Northern Blot Analysis

Total RNA was extracted in 4 M guanidine isothiocyanate (Fluka Chemical Corp., Ronkonkoma, NY), 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M β -mercaptoethanol. 20 μg were fractionated on a 1% agarose-formaldehyde gel (38), transferred to a nylon membrane (Amersham Corp., Arlington Heights, IL) and hybridized to ³²P-labeled probe as described (38). The membranes were stained with methylene blue to ensure equal loading and transfer.

Patients

Patients with the classical T cell-mediated skin diseases allergic contact dermatitis (ACD, $n = 11$) and psoriasis (PSO, $n = 8$) were compared to normal control subjects (NS, $n = 9$). None of the patients had received prior treatment for their skin condition. After informed consent, full thickness 4-mm punch biopsies were obtained from the lesional skin of the patients. In the NS group, 4-mm punch biopsies were taken from the volar aspects of the forearm. Samples were snap frozen immediately and stored at -80°C until further use.

Immunohistochemistry

Frozen skin specimens were embedded in Optimum Cutting Medium (Miles Inc., Elkhart, IN) and 5- μm serial cryostat sections were prepared using a Crycut 2000 (Reichert & Jung, Nußbach, FRG). Air-dried, acetone-fixed frozen sections were stained using a four step immunohistochemical staining protocol (ABC-technique, DAKO): (a) primary mAb (rabbit IgG or mouse IgG); (b) biotin-conjugated goat-anti-mouse IgG, or biotin-conjugated goat-anti-rabbit IgG; (c) peroxidase-conjugated streptavidin (d), and diamino-benzidine as chromogenic substrate. Finally, sections were counterstained with hemalum. Control staining was performed by replacing the primary mAb with isotype-matched control reagents. Staining was evaluated by four independent observers in a blinded fashion using a Zeiss Axioskop, equipped with a MC100 camera system. The opinions of the observers were concordant.

Results

CD44-Ig Fusion Proteins Expressing Alternatively Spliced Exon V3 Are Modified with Heparan Sulfate

Epithelial cells have been shown to express an HS-modified CD44 isoform which contains the alternatively spliced exons V3-V10 (3, 30). Inspection of the amino acid sequence encoded by genomic CD44 revealed that there are six potential GAG attachment sites with the known consensus Ser-Gly. Four of the potential GAG attachment sites are in the "common" region of CD44 and one each in exons V3 and V10. The motif in exon V3 contains a Ser-Gly-Ser-Gly sequence whereas the five other potential GAG sites only contain Ser-Gly (47). Jackson et al. (20) have used Namalwa cell transfectants to characterize the GAG modifications of various CD44 isoforms and showed that CD44 isoforms expressing V3 can be modified with HS.

To confirm these results and explore the possibility of using CD44-Ig fusion proteins to study the ability of HS-modified CD44 to present HS-binding growth factors, four CD44 expression constructs were made (Fig. 1). CD44H-Ig encodes a soluble chimeric protein that contains all the CD44 extracellular common exons (E1-E5, E15, E16) in frame with the hinge, CH2, and CH3 domains of a human IgG₁ (1). CD44E-Ig consists of all the extracellular common exons, as well as exons V8–V10, fused to the same human IgG domains (51). CD44V3, V8–V10-Ig, and CD44V3–V10-Ig both encode for the common extracellular domain exons, the human antibody domains, and the variably spliced exons V3, V8–V10, and V3–V10, respectively.

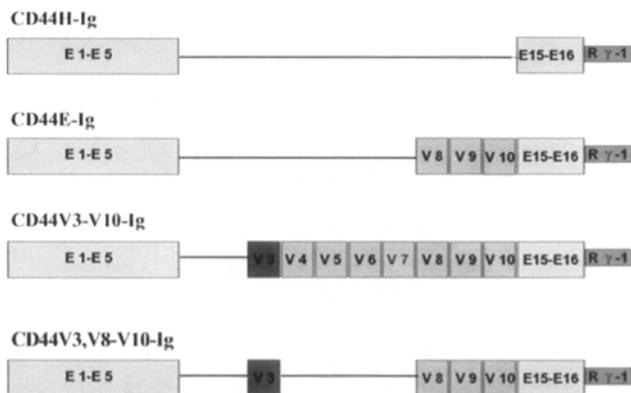


Figure 1. CD44-Ig constructs. Line drawing representation of the four CD44-Ig constructs used in these studies; CD44H-Ig, CD44E-Ig, CD44V3, V8-V10-Ig, and CD44V3-V10-Ig. Exon V3 is shaded since it contains a Ser-Gly-Ser-Gly motif which is a putative site for GAG attachment.

COS cells were transfected with the four CD44-Ig chimeric constructs and metabolically labeled with $^{35}\text{SO}_4^{-2}$. The four purified Ig fusion proteins were either left untreated or digested with chondroitin ABC lyase (ABC), heparitinase (HEP), or both lyases (Fig. 2, *A* and *B*). The majority of the $^{35}\text{SO}_4^{-2}$ is incorporated into GAGs such that the proteins can be analyzed for the loss of label after digestion to determine the type of GAG attached to the proteoglycan. Fig. 2, *A* and *B* show that CD44H-Ig and CD44E-Ig contain sulfate modifications that are sensitive to chondroitin ABC lyase but not to heparitinase digestion. In contrast, when COS cells were transfected with DNA encoding CD44-Ig constructs containing exon V3, the $^{35}\text{SO}_4^{-2}$ modified CD44 recombinant proteins were sensitive to both heparitinase and chondroitin ABC lyase (Fig. 2, *C* and *D*). These data demonstrate that when exon V3 is present, COS cell-produced CD44-Ig fusion proteins are modified with heparan sulfate which is likely attached at the Ser-Gly-Ser-Gly motif in exon V3. Some residual $^{35}\text{SO}_4^{-2}$ label remains after digestion with heparitinase and chondroitin ABC lyase which is most likely keratin sulfate which is known to decorate CD44 (51).

***b*-FGF and HB-EGF Bind to HS-modified CD44 Isoforms**

To directly examine the ability of HS-modified CD44 isoforms to bind heparin-binding growth factors, *b*-FGF, AR, and HB-EGF were iodinated and used in ligand-binding assays. We chose *b*-FGF for this study since its interactions with HS and HS-modified proteoglycans have been thoroughly characterized. An HS-modified CD44 isoform is abundantly expressed in keratinocytes and the potential biological role for CD44 in keratinocyte growth seemed intriguing (3, 12). Therefore, AR and HB-EGF, two heparin-binding proteins, were analyzed since they are autocrine factors for keratinocytes (7, 13, 39, 49). OM, a non-HS-binding protein, was also iodinated and used in this assay as a control.

CD44-Ig, CD44E-Ig, CD44V3V8-V10-Ig, and CD44V3-V10-Ig were produced in COS cells and their ability to bind *b*-FGF, HB-EGF, AR, and OM was examined. Shown in Fig. 3 *A* are the different CD44 isoforms electrophoresed, trans-

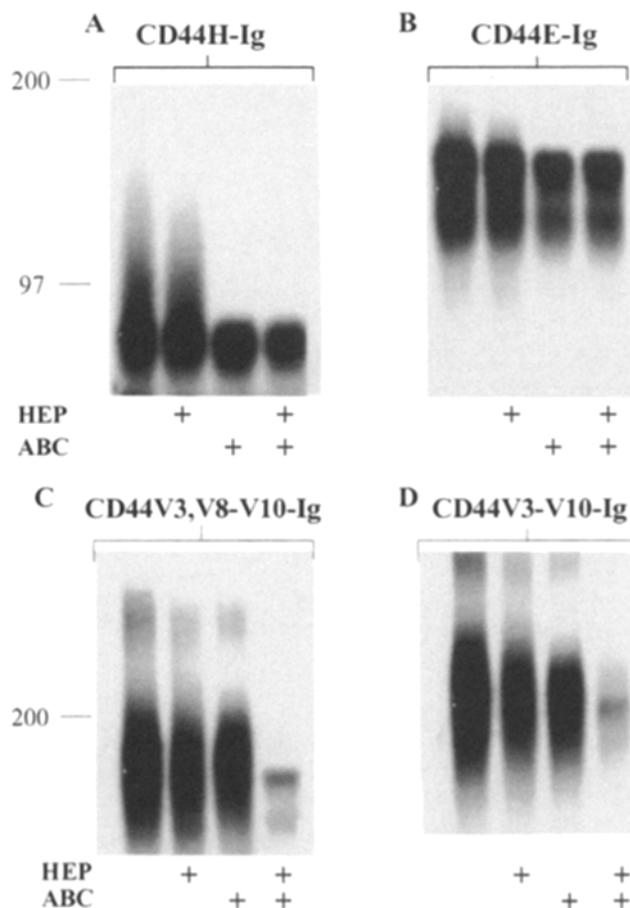


Figure 2. GAG modification of CD44 isoforms. (*A*) $^{35}\text{SO}_4^{-2}$ -labeled CD44H-Ig, (*B*) CD44E-Ig, CD44V3, (*C*) V8-V10-Ig, and (*D*) CD44V3-V10-Ig were recovered from the supernatant of COS cell transfectants, purified, and divided equally into four aliquots. One aliquot was left untreated, the others were digested for 1 h with HEP, chondroitin ABC lyase (ABC), or with both. The proteins were then resolved by SDS-PAGE and analyzed by radiography.

ferred to a nitrocellulose membrane, and probed with ^{125}I -*b*-FGF. HS-modified CD44 isoforms containing variably spliced exon V3 bound the ^{125}I -*b*-FGF (Fig. 3, lanes 3 and 5). Digestion with heparitinase abolished ^{125}I -*b*-FGF binding (lanes 4 and 6). CD44H-Ig and CD44E-Ig which are decorated with chondroitin sulfate (CS) did not bind ^{125}I -*b*-FGF (lanes 1 and 2). These data indicate that the CD44 isoforms modified with HS but not CS can bind *b*-FGF. An identical experiment carried out with ^{125}I -HB-EGF also demonstrated that the interactions between CD44 and HB-EGF were HS dependent (Fig. 4). The specificity of the CD44/*b*-FGF interaction was examined by the following competition assays. Probing of the ligand blots in the presence of 300 $\mu\text{g}/\text{ml}$ of heparin effectively blocked both ^{125}I -*b*-FGF (Fig. 3 *B*) and ^{125}I -HB-EGF (Fig. 4 *B*) binding to HS-modified CD44 isoforms. Approximately 100-fold excess unlabeled *b*-FGF was used to compete the binding of ^{125}I -*b*-FGF to the HS-modified CD44 isoforms; some nonspecific background binding can be seen in lane 1 (Fig. 3 *C*). To further examine the specificity of these interactions, an ELISA approach was used which demonstrated a concentration and HS dependents for CD44 and *b*-FGF interactions

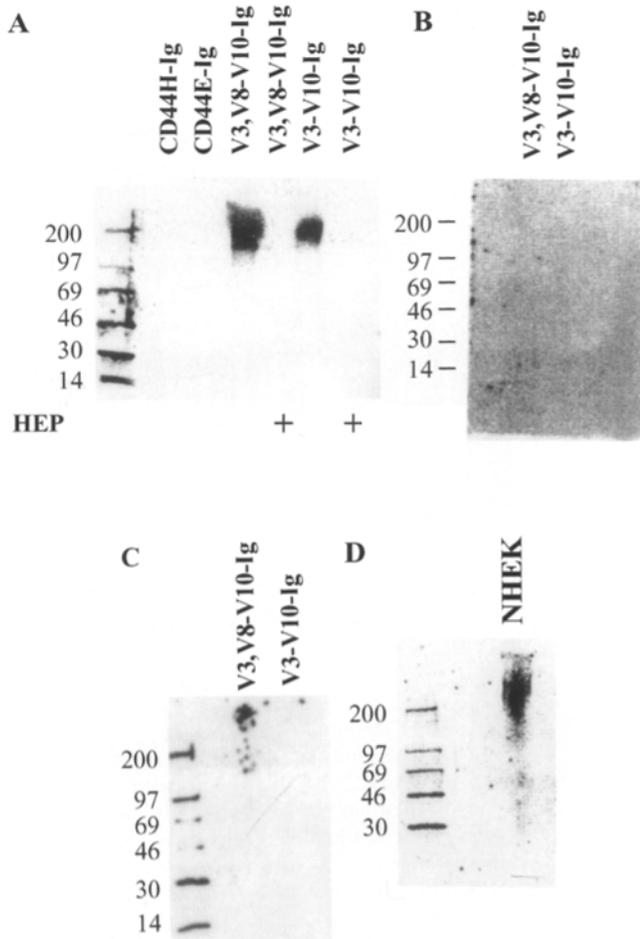


Figure 3. Binding of b-FGF to HS-modified CD44. (A-D) Purified CD44 proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, incubated with ^{125}I -b-FGF, washed, and analyzed by radiography. (A) CD44H-Ig, CD44E-Ig, CD44V3, V8-V10-Ig, and CD44V3-V10-Ig alone or treated with HEP (+). (B) CD44 V3, V8-V10-Ig, and CD44 V3-V10-Ig were analyzed for binding of ^{125}I -b-FGF in the presence of 300 $\mu\text{g}/\text{ml}$ of heparin. (C) CD44V3, V8-V10-Ig, and CD44V3-V10-Ig were analyzed for binding ^{125}I -b-FGF in the presence of a 100-fold excess of unlabeled b-FGF. (D) CD44 was immunoprecipitated from NHEK and analyzed for binding ^{125}I -b-FGF.

(Fig. 5). CD44V3V8-V10-Ig was immobilized onto a 96-well plate and the binding of biotin-conjugated b-FGF was examined. The binding of b-FGF was concentration dependent (*open bars*), and effectively competed by 3 mg/ml of heparin (*striped bar*). Furthermore, digestion of CD44V3V8-V10-Ig with heparitinase also abolished the binding (*black bars*).

Normal human epithelial keratinocytes (NHEK) have been shown to express a HS-modified CD44 isoform (12). To show that the ability of HS-modified CD44 to bind b-FGF was not a property unique to the fusion protein, CD44 immunoprecipitated from NHEK was tested for its capacity to interact with ^{125}I -b-FGF. Fig. 3 C shows that CD44 produced by NHEK can bind ^{125}I -b-FGF. This demonstrates that HS-modified CD44 expressed either as a recombinant immunoglobulin fusion protein in COS cells or as an endogenous membrane bound protein in NHEK is able to bind b-FGF.

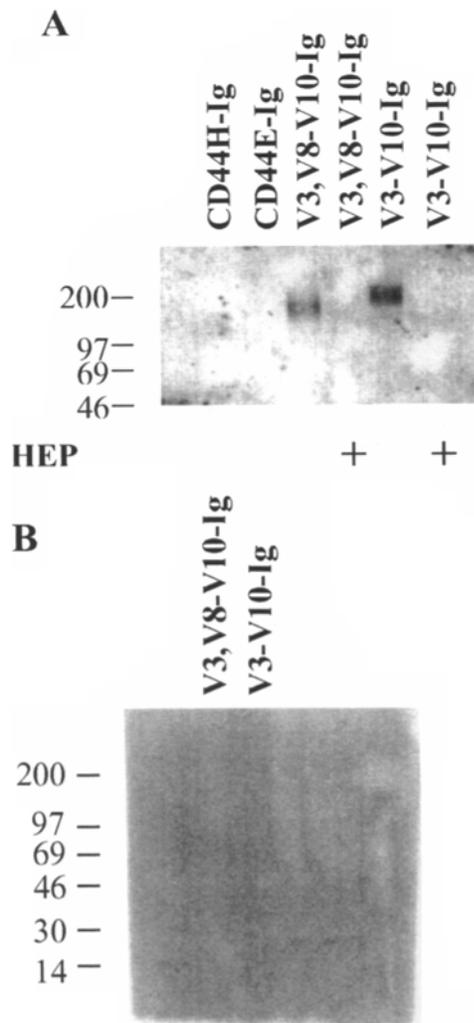


Figure 4. Binding of HB-EGF to HS-modified CD44. (A-B) Purified CD44 proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, incubated with ^{125}I -Hb-EGF, washed, and analyzed by radiography. (A) CD44H-Ig, CD44E-Ig, CD44V3, V8-V10-Ig, and CD44V3-V10-Ig alone or treated with HEP (+). (B) CD44V3, V8-V10-Ig, and CD44V3-V10-Ig were analyzed for binding of ^{125}I -HB-EGF in the presence of 300 $\mu\text{g}/\text{ml}$ of heparin.

^{125}I -AR and ^{125}I -OM did not bind to any of the CD44 isoforms tested (data not shown). To determine if AR was still able to bind HS after iodination, 2 μCi of all four of the iodinated proteins were incubated with Sepharose and heparin Sepharose beads for 1 h, and then washed. ^{125}I -b-FGF, ^{125}I -HB-EGF, and ^{125}I -AR bound substantially more to the heparin Sepharose demonstrating that the iodination had not inhibited the ability of AR to bind heparin, whereas ^{125}I -OM did not show a similar increase in binding to the heparin Sepharose (Table I). While ^{125}I -AR is able to bind to the heparin Sepharose beads but not the HS-modified CD44, it cannot be ruled out that the ^{125}I -AR is able to function in a ligand blot format. However, the experiments with AR were repeated numerous times and strongly suggest that it does not bind to the HS attached to CD44 made in COS cells.

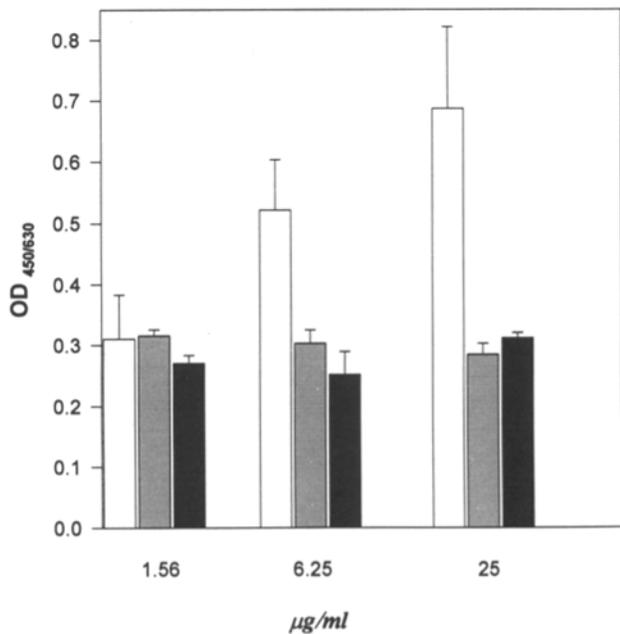


Figure 5. Binding of b-FGF to HS-modified CD44 by ELISA. CD44V3V8-V10-Ig (open bars) bound biotin-conjugated b-FGF in a concentration-dependent manner. (Striped bars) Binding is blocked in the presence of 3 mg/ml heparin. (Black bars) Heparinase digestion of CD44V3V8-V10-Ig abolished b-FGF binding.

In Vitro-activated Endothelial Cells Express CD44

To examine the proposed role of CD44 in the presentation of HS-binding chemokines to leukocytes along activated vascular endothelium, a number of experiments were conducted to determine which CD44 isoforms are expressed by activated endothelial cells and if these CD44 isoforms are modified with GAG. The first level of analysis involved an examination of the expression of CD44 in cultured endothelial cells. It had been previously reported by Mackay and colleagues that endothelial cells express low levels of CD44 which can be upregulated after activation with TNF- α (37). In our hands, cultured HUVEC constitutively express high levels of CD44, the expression of which is not further upregulated after treatment for 6 h with TNF- α , IL-1, LPS, INF- γ , thrombin, or histamine (Fig. 6). Also, no changes in the levels of CD44 expression were observed when the cells were treated with TNF- α for various time periods between 30 min and 96 h (Fig. 6). These experiments were done with two different donors; in both cases we observed that under our culture conditions CD44 is abundantly expressed by HUVEC cells and that additional stimulation does not result in higher levels of CD44 expression. This difference might be due to differences in the growth factors added to the media

Table 1. Binding of Iodinated Proteins to Heparin

cpm	¹²⁵ I-b-FGF	¹²⁵ I-AR	¹²⁵ I-HB-EGF	¹²⁵ OM
Sepharose	1,761	6,137	18,279	19,943
Sepharose + Heparin	63,932	83,815	173,105	13,632

2 μ Ci of each iodinated protein was added to Sepharose and heparin Sepharose beads for 1 h, and then washed and counted.

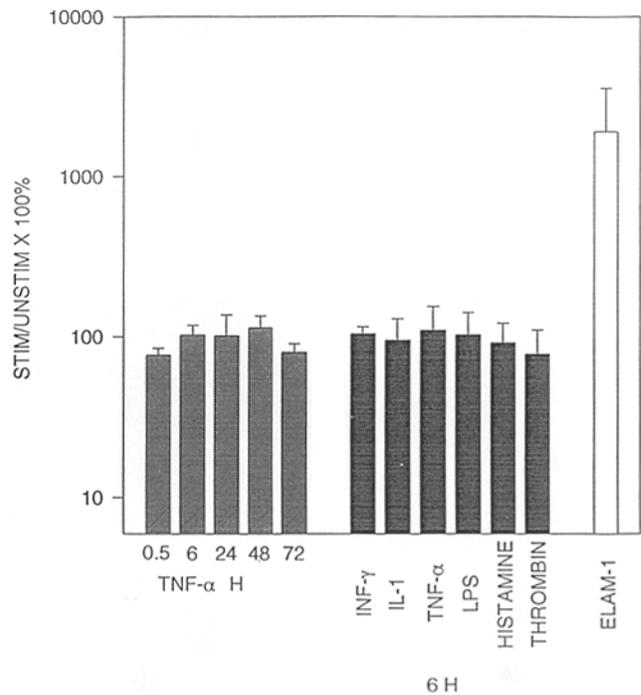


Figure 6. Expression of CD44 protein by cultured endothelial cells. HUVEC were cultured in the presence of the indicated proinflammatory mediators, lifted with EDTA, and stained with isotype controls, anti-CD44 mAb A3D8, and anti-ELAM-1. The bar graph sets the expression level of the unstimulated cells to 100%. Unstimulated cells and the stimulated cells expressed abundant CD44. Unstimulated HUVEC did not express ELAM-1, but upon activation was strongly upregulated.

used to maintain these primary cells in culture in different laboratories. However, it should be noted that these cells have not become refractory to activation by these proinflammatory mediators which are still able to direct the expression of other inducible cell adhesion molecules. For example, TNF- α treatment of these cells results in E-selectin expression (Fig. 6). Taken together, the results of Mackay et al. (37) and the data presented herein show that activated endothelial cells express CD44.

Northern blot analysis of human aortic endothelial cells (HAEC) activated with b-FGF, α -FGF, TGF- β , or TNF- α for 48 h confirmed that in our culture conditions CD44 was abundantly expressed before and after stimulation in endothelial cells (data not shown).

Activated Endothelial Cells Do Not Express CD44 Isoforms Containing V3

As demonstrated by Jackson et al. (20) and corroborated above, CD44 isoforms containing exon V3 can be modified with HS. Based on the proposed role of HS-modified CD44 in the presentation of HS-binding chemokines to lymphocytes by activated endothelial cells, we examined the ability of activated endothelial cells to express CD44 isoforms containing exon V3 by flow cytometry using an anti-V3 specific antibody. HUVEC cells were stained with either an anti-CD44 mAb which recognizes all CD44 isoforms or with an antibody which recognizes an epitope present only in V3 and analyzed by flow cytometry. As shown in Fig. 7 A, HUVEC

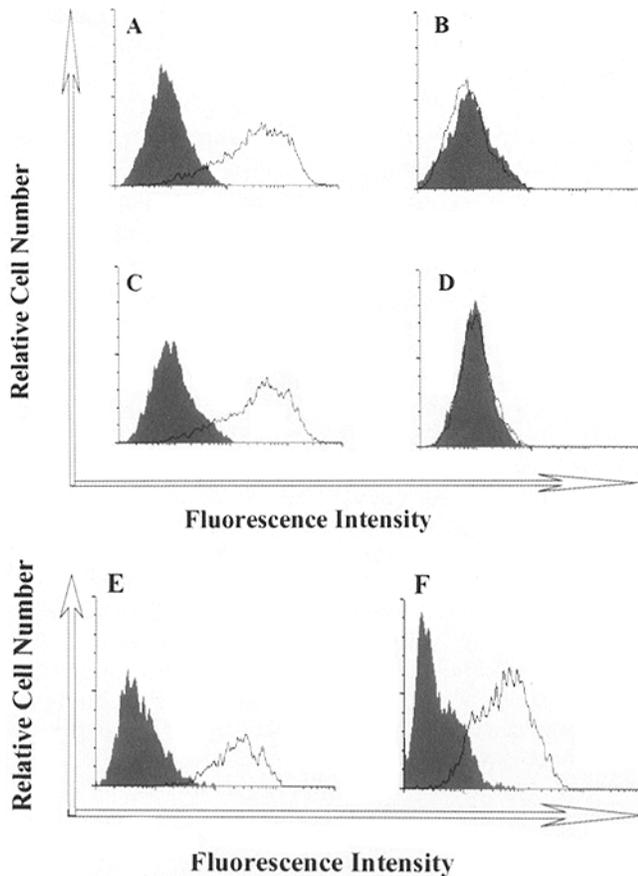


Figure 7. Cell surface expression of CD44 isoform encoding V3 by cultured epithelial and endothelial cells. Line drawings representing flow cytometry profiles of cells expressing CD44. (A–D) Cultured endothelial cells from pooled donors were stained with either isotype-matched controls (*shaded profile*) and a mAb for either the “constant” region of CD44 (A and C) or a mAb specific for CD44 exon V3 (B and D). A and B represent profiles from unstimulated cells, while C and D are FACS profiles from cells stimulated with TNF- α for 48 h. NHEK were stained with either a mAb to the constant region of CD44 (E) or with a mAb for CD44 exon V3 (F).

express abundant levels of CD44 but do not bind to the antibody recognizing exon V3 (Fig. 7 B). Treatment with TNF- α for 48 h did not change the binding profiles of these two antibodies (Fig. 7, C and D). Similar antibody-binding experiments on keratinocytes showed that these cells express abundant levels of CD44 which contain exon V3 (Fig. 7, E and F). These results show that activated endothelial cells do not express CD44 isoforms containing V3.

Analysis of CD44 RNA Transcripts in Endothelial Cells

To investigate the presence of RNA transcripts encoding different CD44 isoforms in activated endothelial cells, RT-PCR was carried out. Since NHEK are known to express CD44 isoforms with alternatively spliced exons, we used these cells as a control throughout our experiment. RT-PCR was carried out on RNA isolated from NHEK, human mammary epithelial cells (HMEC), HAEC, and HUVEC using PCR primers located in the common exons E3 (forward primer) and E16 (reverse primer, Fig. 8 A). As shown in Fig.

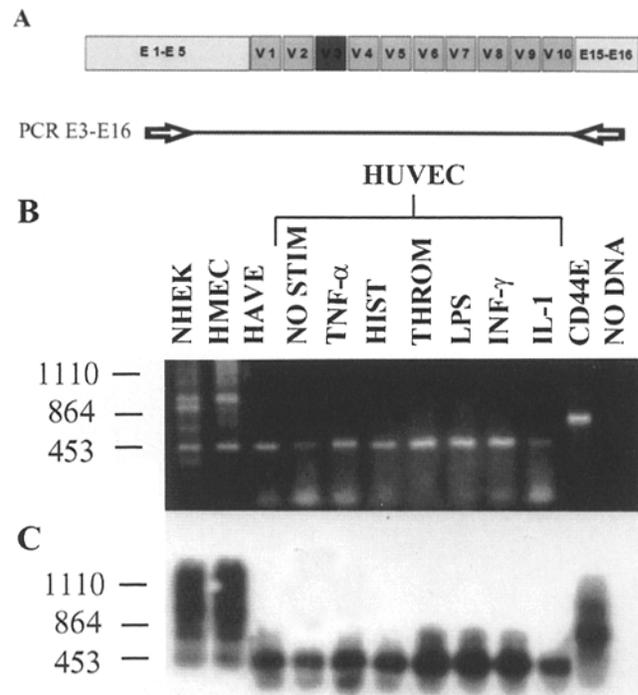


Figure 8. Expression of CD44 variably spliced exons by cultured epithelial and endothelial cells. (A) Line drawing representation of the extracellular domain exons of CD44. E1–E5 and e15–E16 represent the common exons shared by all CD44 isoforms. V1–V10 represents potential alternatively spliced exons. Arrows represent the forward (CD44E3-FP) and reverse (CD44E16-RP) primers that were used to generate RT-PCR bands shown in B. (B) RT-PCR was carried out on the indicated cell types and proinflammatory mediators were added to HUVEC for 6 h, the PCR products were fractionated on a 2% agarose gel and stained with ethidium bromide. (C) The PCR products were transferred to a nylon membrane and probed with a 32 P-random primed probe to CD44E.

8 B, PCR products obtained from NHEK form a DNA ladder which can be detected both in the ethidium bromide-stained gel (Fig. 8 B) and in the corresponding DNA blot probed with a 32 P-labeled CD44E cDNA (Fig. 8 C). The same pattern of CD44 isoform expression is seen in normal HMEC. These data confirm that NHEK express CD44 isoforms containing alternatively spliced exons and demonstrate that HMEC cells also express alternatively spliced CD44 isoforms. In contrast, cultured HAEC and HUVEC generated a single-sized DNA of 543 bp, the predicted size for CD44H (Fig. 8, B and C). The PCR product was purified and sequenced to confirm that it did not contain any alternatively spliced exons (data not shown). As a second control, a CD44E clone was used for PCR analysis and generated a band of 894 bp, the expected size for a CD44 isoform containing the variably spliced exon V8–V10 (Fig. 8, B and C). Stimulation of HUVEC for 6 h with a number of different proinflammatory mediators (TNF- α , Histamine, Thrombin, LPS, IFN- γ , IL-1) did not induce the expression of alternatively spliced CD44 isoforms (Fig. 8, B and C).

RT-PCR was used to specifically look for the expression of CD44 transcripts containing exon V3 in the following cell types: HUVEC, HMEC, NHEK, a fibroblast cell line WI-38, and HT-1080 cells (fibrosarcoma cells). Two RT-PCR ex-

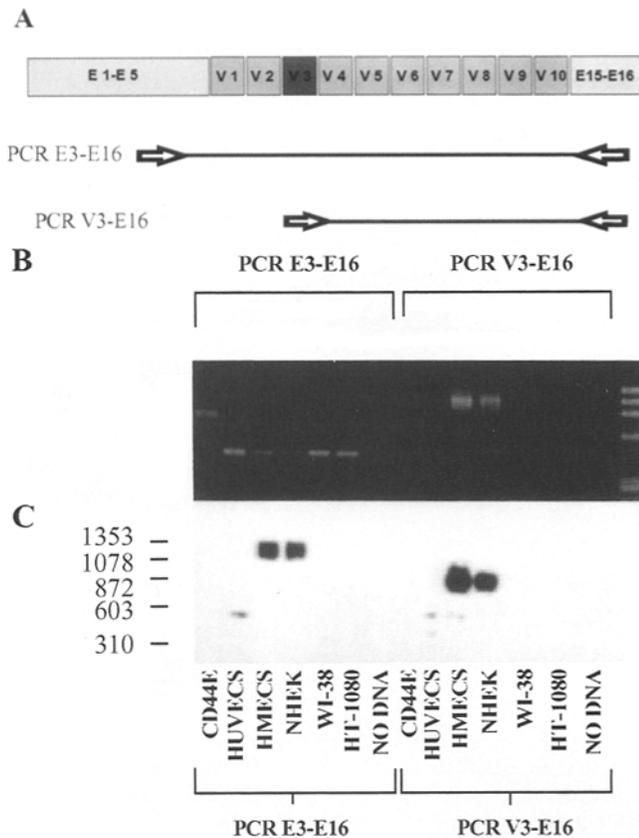


Figure 9. Expression of CD44 transcripts encoding V3 by cultured epithelial and endothelial cells. (A) Line drawing representation of the extracellular domain exons of CD44. E1-E5 and E15-E16 represent the common exons shared by all CD44 isoforms. V1-V10 represents potential alternatively spliced exons. Arrows represent the three different primers used for RT-PCR: two of the primers are to common exons CD44E3-FP, CD44E16-RP; and forward primer CD44V3-FP is to the variably spliced exon V3. (B) RT-PCR products were run on a 2% agarose gel and stained with ethidium bromide. (C) The gel was transferred to a nylon membrane and probed with a ^{32}P -end-labeled oligonucleotide to V3.

periments were carried out, the first using the primers located in E3 and E16 described in Materials and Methods; the second using a primer located in V3 (forward primer) and the E16 (reverse primer) also described in Materials and Methods (Fig. 9 A). In both cases the presence of V3 in the PCR products was examined by Southern blot using a ^{32}P -labeled probe specific to exon V3. Fig. 9, B and C show that exon V3 is present in the PCR product generated from NHEK and HMEC. PCR primers E3/E16 gave rise to PCR products with the predicted size for CD44H (Fig. 9 B) using RNA isolated from HUVEC, WI-38, and HT-1080 cells. These PCR products did not bind to the V3 specific probe (Fig. 9 C). PCR primers V3/E16 failed to generate PCR products when RNA from HUVEC, WI-38, and HT-1080 cells were used as determined by ethidium bromide staining (Fig. 9 B) and probing with the radiolabeled V3 specific probe (Fig. 9 C). These same primers gave rise to the expected PCR product using RNA isolated from NHEK and HMEC cells (Fig. 9 B) which bound to the V3 specific probe (Fig. 9 C). As expected, a control sample using CD44E cDNA gave rise to a PCR product of the expected size when

the E3/E16 primers were used, which did not bind to the V3 specific probe, and failed to generate PCR products when the V3/E16 primers were used.

Expression of CD44 Isoforms Containing Variably Spliced Exons in Inflamed Skin

In the experiments described above we used cultured endothelial cells and keratinocyte cells to examine the expression of CD44 isoforms containing variably spliced exons. To determine if these observations reflect what is found *in vivo*, we examined the expression of CD44 containing variably spliced exons (CD44V) in keratinocytes, endothelial cells, monocytes, and dendritic cells found in inflamed skin by immunohistology. Tissue from two classical T cell-mediated inflammatory skin diseases, *i.e.*, allergic contact dermatitis and psoriasis were examined for CD44V-expression. No CD44V staining could be detected on dermal endothelial cells using either a polyclonal Ab directed against all CD44V, including V3 (15), or mAb-specific for CD44-V5 and CD44-V6 (29) (Fig. 10, A-C). This lack of CD44V expression was observed even in the presence of dense perivascular lymphocytic infiltrates (Fig. 10) and was identical to that of normal skin (data not shown). In contrast, we found that these reagents clearly detect CD44 isoforms containing variably spliced exons expressed by epidermal keratinocytes and activated macrophages and dendritic cells in the same specimen (Fig. 10) with keratinocytes expressing abundant amounts of a CD44 isoform containing V3. In agreement with what had been previously reported by Heider *et al.* (14), we find that endothelial cells express low levels of a CD44 which is recognized with an antibody which binds to an epitope in the common exons (Simon, J., and E. Tanczos, unpublished).

GAG Modification of Endothelial Cell CD44

Finally, we examined whether CD44 expressed on HUVEC is modified with glycosaminoglycans. In this experiment NHEK, HT-1080 cells, and HUVEC were metabolically labeled with $^{35}\text{SO}_4^{-2}$ for 4 h, immunoprecipitated with an anti-CD44 mAb, analyzed by SDS-PAGE, and followed by autoradiography. No detectable $^{35}\text{SO}_4^{-2}$ modified CD44 was obtained from endothelial cells (data not shown) but very strong signals were detected from CD44 immunoprecipitated from HT-1080 cells (Fig. 11 B) and NHEK (Fig. 11 C). Extending the labeling time from 4 h to 16 h for the endothelial cells resulted in the detection of a very low amount of $^{35}\text{SO}_4^{-2}$ -labeled CD44 when the gel was exposed to film for a prolonged time period (Fig. 11 A). Digestion of the $^{35}\text{SO}_4^{-2}$ -labeled CD44 obtained from HT-1080 cells with chondroitin ABC lyase removed all of the label suggesting that in these cells, which express predominantly CD44H, CD44 is modified with CS (Fig. 11 B). Digestion of CD44 obtained from the NHEK with chondroitin ABC lyase and heparitinase removed 70% of the label suggesting that the CD44 expressed by these cells, which contains V3, is modified with both CS and HS (Fig. 11 C). In contrast, digestion of CD44 immunoprecipitated from HUVEC with both chondroitin ABC lyase and heparitinase did not result in any detectable loss of label even after overexposure of the gel (Fig. 11 A). Treatment of the endothelial cells with TNF- α , histamine, IL-1, LPS, INF- γ , and thrombin for 48 h

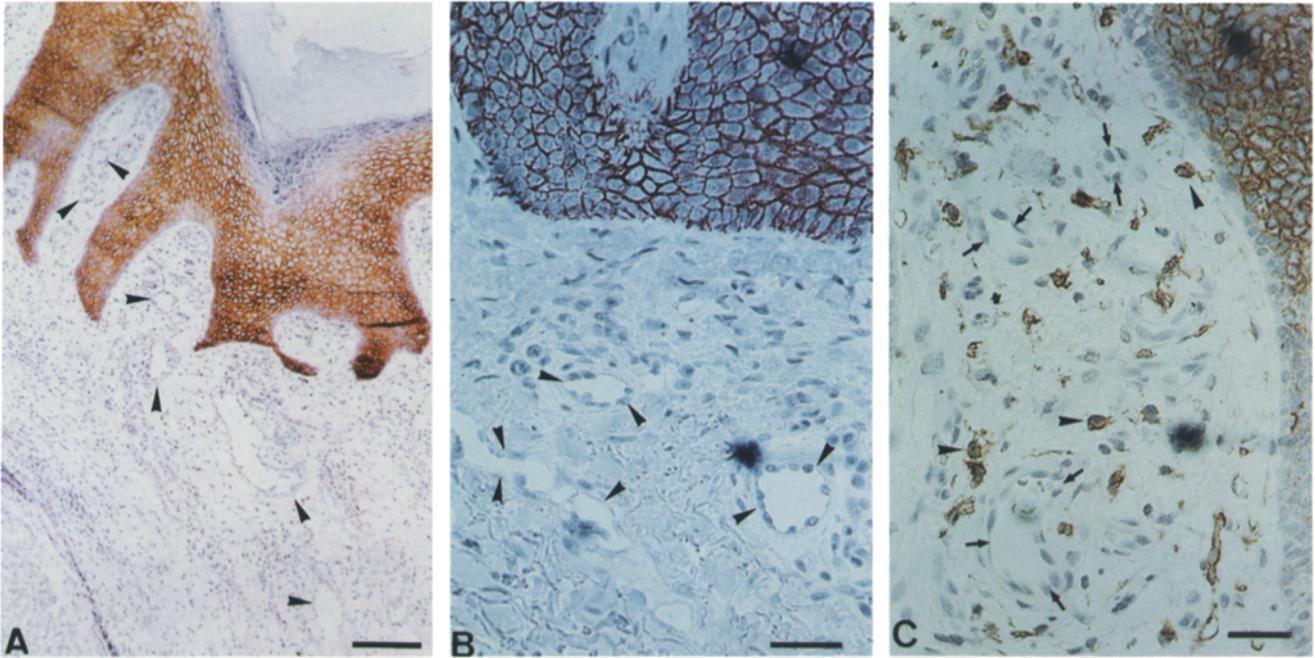


Figure 10. Expression of CD44 with variably spliced exons by keratinocytes, endothelial cells, monocytes, and dendritic cells in inflamed skin. (A) Acute allergic contact dermatitis: CD44V is expressed by keratinocytes in the epidermis but not by dermal endothelial cells (arrowheads) (CD44V poly); (B) allergic contact dermatitis: CD44V5 is expressed by keratinocytes in the epidermis but not by dermal endothelial cells (arrowheads) (VFF8); and (C) psoriasis: CD44V6 is expressed by keratinocytes in the epidermis and by dermal macrophages and dendritic cells (arrowheads) but not by dermal endothelial cells (small arrows) (VFF7). Bars: (A) 100 μm ; (B and C) 63 μm .

and labeling with $^{35}\text{SO}_4^{-2}$ for 16 h also resulted in only a minimal amount of label incorporated into the immunoprecipitated CD44 (data not shown).

Discussion

Expression of different CD44-Ig constructs in COS cells has allowed us to establish that CD44 isoforms containing variably spliced exon V3 can be modified with HS. These results are in agreement with the findings of Jackson et al. (20) who extensively characterized the GAG modifications of different CD44 isoforms. Selected serine residues are used by xylosyltransferase to initiate GAG chain elongation, and attempts to define consensus recognition sequences surrounding the serines have been made (for review see 26). Ser-Gly-X-Gly preceded by acidic residues is recognized by xylosyltransferase in a number of GAG-substituted proteins, and synthetic peptides. There are also a number of proteins that do not contain the above consensus at sites of GAG attachment; for example, some proteins have GAGs attached to serines in the context of only Ser-Gly. While there are six potential GAG attachment sites in CD44, five of the sites constitute the most minimal consensus Ser-Gly and some of these sites are modified by chondroitin sulfate. In contrast, exon V3 has a Ser-Gly-Ser-Gly motif. The data presented herein and that presented by Jackson et al. (20) show that this site can be modified with heparan sulfate in a variety of cell types. The relationship between the protein backbone and the type of GAG added is presently not understood, but there is increasing evidence that there are determinants that reside in the proteoglycan core protein which influence this

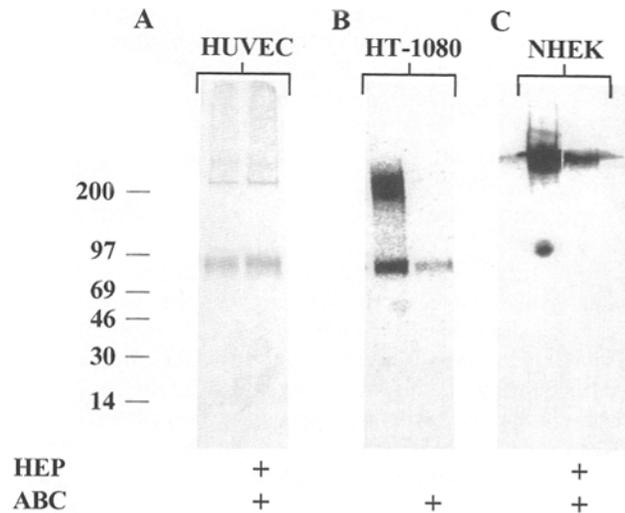


Figure 11. GAG modification of CD44 expressed by cultured epithelial and endothelial cells. (A) HUVEC were labeled for 16 h with $^{35}\text{SO}_4^{-2}$, immunoprecipitated with an anti-CD44 mAb (pgp-1), the sample was divided in two, half of the sample was digested with both HEP and chondroitin ABC lyase (ABC), and then both samples were run on SDS-PAGE and analyzed by radiography. (B) HT-1080 cells were labeled for 4 h with $^{35}\text{SO}_4^{-2}$, immunoprecipitated with an anti-mAb (pgp-1), the sample was divided in two, half of the sample was digested with chondroitin ABC lyase (ABC), and then both samples were run on SDS-PAGE and analyzed by radiography. (C) NHEK were labeled for 4 h with $^{35}\text{SO}_4^{-2}$, immunoprecipitated with an anti-CD44 mAb (pgp-1), the sample was divided in two, half of the sample was digested with both HEP and ABC, and then both samples were run on SDS-PAGE and analyzed by radiography.

process (28, 40). These findings parallel those reported by Kokenyesi and Bernfield who present data that suggest that the Ser-Gly-Ser-Gly motif in syndecan is modified with HS while the Ser-Gly motifs are modified with CS (28).

Understanding the multiple functions of the various CD44 isoforms is the focus of intensive research. The role of proteoglycans as reservoirs of and presenters of glycosaminoglycan-binding growth factors, cytokines, and chemokines is well established (for review see 21, 27, 55, 63). Recently, Shaw and colleagues have shown that GAG-modified CD44 isolated from peripheral blood monocytes is able to bind and present MIP-1 β to T cells and induce their ability to bind VCAM-1 in vitro (53). Here we show that only CD44 isoforms containing variably spliced exon V3 are modified with HS and can present some, but not all, HS-binding growth factors. More specifically, we found that HS-modified CD44 can bind to b-FGF, and HB-EGF but not to AR. Only CD44 isoforms containing exon V3 become decorated with HS. Heparitinase digestion and free heparin abolished the interaction between the HS-modified CD44 isoforms and the growth factors demonstrating that these interactions are specifically mediated by HS. Furthermore, other GAGs such as CS do not participate in the interactions since CD44 isoforms modified by CS did not bind to any growth factor tested. The culmination of numerous approaches used to identify the heparin-binding domain of b-FGF has demonstrated that the binding domain is composed of a discontinuous set of amino acids, and that the majority of the binding free energy is contributed by hydrogen bonding and van der Waals packing (57) providing for very specific interactions. In addition, synthetic peptides and mutagenesis studies have been used to begin to identify the heparin-binding domain of HB-EGF (58), whereas the AR heparin-binding domain at this point can only be speculated. To fully understand the specificity of binding by these three proteins to the heparan sulfate on CD44 will require a complete characterization of their heparin-binding domains.

The structural diversity of the HS moieties added to proteins is well documented; greatly varied is the number of glucuronic acid moieties epimerized to iduronic acid, and the degree and placement of sulfates, giving rise to very heterogeneous molecules (for review see 26). This diversity in HS gives rise to domains with unique structures that provide specificity for interacting with different HS-binding proteins. The best studied example of the specificity of the interactions between a heparin-binding protein and different heparan sulfate structures is the interaction between antithrombin III and its pentasaccharide target. These studies have shown that a small subset of HS-modified proteoglycans contain the appropriate pentasaccharide (23, 36). Furthermore, the binding affinity of antithrombin III for different HS-proteoglycans made in the same cell can vary greatly, suggesting that structural determinants in the protein backbone dictate the type of GAG biosynthesis and modifications that take place (40). The specificity of the interaction of b-FGF with HS was first demonstrated by showing that the extent of b-FGF binding depended upon the HS source; HS from pig aorta bound substantially more to b-FGF than did HS from mouse Engelbreth-Holm-Swarm tumors (11). The identification of the minimal hexasaccharide required for maximal binding confirms the specificity of the interactions (19, 60, 61). These results are consistent with our observation that

COS cell-produced CD44 isoforms containing V3, which are modified with HS, can differentially interact with various HS-binding proteins. Our finding that HS-modified CD44 produced by keratinocytes can bind to b-FGF further suggests that in these cells CD44 may participate in the presentation of HS-binding growth factors, chemokines and/or cytokines, and provide further activation signals to leukocytes during an inflammatory response in the skin. In addition, the binding of HB-EGF to HS-modified CD44 suggests an important role for CD44 in keratinocyte growth since HB-EGF is a keratinocyte autocrine growth factor (13).

The ability of GAG-modified CD44 to bind and present HS-binding proteins to leukocytes has led to the proposition that this proteoglycan might play a role in the presentation of chemokines by inflamed vascular endothelial cells. FACS, Northern blot, and PCR analysis demonstrated that cultured endothelial cells express abundant CD44H. However, we found no evidence that CD44 isoforms containing variably spliced exons were expressed by these cells even after treatment with proinflammatory mediators such as TNF- α , IL-1, INF- γ , histamine, thrombin, or LPS. This observation is consistent with our finding that in the inflamed skin from patients with ACD or PSO vascular endothelial cells do not express CD44 isoforms containing variably spliced exons. These results in conjunction with the data described by Heider et al. (14) who showed that endothelial cells in normal tissue samples express low levels of CD44 containing no variably spliced exons, and the data of Mackay et al. (37) which showed that CD44 expression could be upregulated after endothelial cell activation suggest that only expression of CD44H is upregulated after endothelial cell activation in vivo. Furthermore, we also showed that exon V3, which provides the required sequence motif for the modification of CD44 with HS, was not expressed by activated endothelial cells. These results suggest that CD44 is not one of the proteoglycans involved in the presentation of HS-binding chemokines to leukocytes on inflamed vascular endothelium. This interpretation is supported by the identification of the major HS-modified proteoglycans from HAEC and HUVEC which included syndecan, glypican, fibroglycan, and perlecan, but not CD44 (40).

Although CD44H is not modified with HS it can be modified with CS. Metabolic-labeling experiments with $^{35}\text{SO}_4^{-2}$ demonstrated that CD44H expressed by activated endothelial cells is not extensively modified with GAGs. Although we cannot rule out the possibility that endothelial cell proteoglycans are not easily labeled using this protocol, our data suggest that on activated endothelial cells CD44H is not a GAG-modified proteoglycan. This data is supported by the observations of Bourguignon et al. (2) who showed by cell surface labeling endothelial cells with ^{125}I that CD44 is expressed as a 116-kD glycoprotein, and not as larger more diffuse molecules characteristic of GAG modifications.

In summary, this report provides direct evidence that CD44 isoforms containing variably spliced exon V3 can bind to a subset of HS-binding growth factors. A more thorough examination of the cell types that express CD44V3 isoforms and the HS-binding proteins which CD44 presents during development, tumor growth and metastasis, and other hyper proliferative diseases such as inflammatory skin diseases will likely reveal that CD44 contributes a critical function for these biological processes.

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